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By screening cDNA expression libraries with autologous breast cancer patient serum, we have identified and characterized two new gene products (*Ngp-1* and *LMO4*), both of which appear to play vital roles in cellular growth and differentiation. The first autoantigen isolate *Ngp 1* is a nucleolar GTP-binding protein which appears to be a vital component of the pre-mRNA processing machinery. *LMO4* is partially homologous to a known oncogene, *LMO1*, and interacts with other gene products involved in gene regulation and signal transduction. Our observation that the expression of *LMO4* transcripts in breast tumors differs from that in other tissues, and that *LMO4* interacts with a protein involved in estrogen receptor signal transduction, hint at a possible role of *LMO4* in breast cancer, and merits further investigation. Furthermore, structural features of the *LMO4* cDNA sequence all predict that this gene plays a vital role in the life of the organism.

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INTRODUCTION

Tumor growth is associated with the expression of mutated gene products, inappropriate gene expression, and the breakdown of tissue architecture, leading to the exposure and release into the peripheral circulation of sequestered antigens (1,2). Whether these circulating, mutated or newly displayed tumor-associated antigens elicit an autologous humoral immune response in the breast tumor patient is of vital interest. Isolation, identification and characterization of novel breast tumor associated autoantigens might yield new insights into the disease process, and moreover, may be developed into diagnostic screening tests and potential targets for immunotherapy.

The screening of cDNA expression libraries with autologous patient serum is a powerful technique, which has been used successfully for the identification of autoimmune disease antigens (3), and which we have adapted for the identification of autoantigens in cDNA libraries made from breast tumor mRNA. After screening cDNA libraries, derived from primary ductal breast carcinomas with autologous patient serum, we have detected and isolated three immunoreactive cDNA clones, all three of which are newly discovered gene products. The first autoantigen isolate *Ngp 1* has been characterized and is a nucleolar GTP-binding protein which appears to be a vital component of the pre-mRNA processing machinery. The predicted amino acid sequence of the second clone (tentatively named *LMO4*) contains two LIM domain motifs and bears a 60% homology in this region to a known oncogene, *LMO1*. Our studies have identified novel proteins that appear to play vital roles in the regulation of cellular growth, and may help in the understanding of normal cellular proliferation as well as malignancy.

BODY

Most of our effort for the past year has focused on characterizing and defining the role of our second breast tumor autoantigen isolate (tentatively named *LMO4*), which is a newly discovered gene. We chose to focus on LMO4 because we found it to be related to a group of known oncogenes, and our observations indicate that it is an important regulatory element in cellular growth and differentiation and possibly relevant to breast cancer. Work is continuing on our first autoantigen isolate *Ngp-1*, the nucleolar GTP-binding protein (4), to identify other proteins that interact with it using the yeast two-hybrid vector system (5). In a study characterizing the networks of interactions between yeast proteins (6) it was reported that the yeast homologue of *Ngp-1* interacted with six other yeast proteins, three of which are known pre-mRNA splicing factors. Furthermore, the *Ngp-1* homologue was found to occupy a central role in this regulatory mechanism, and that disruption of the *Ngp-1* open reading frame (ORF) was lethal to the organism. We have not been able to further characterize the third autoantigenic clone (*Auag3*) which, with the exception of expressed sequence tags in the databases, shows no homology to any known gene.

The 5′ end 600 bases of the *LMO4* sequence proved to be highly GC rich (74%), with some stretches exceeding 90%. Formamide containing sequencing gels had to be used to obtain an accurate sequence. The high GC content of the 5′ portion of the cDNA explains the under-representation of full length clones in cDNA libraries, since it would impart a high degree of secondary structure, interfering with reverse transcription. Standard PCR reactions were also ineffective across this region, and special formulations for melting high GC DNA had to be used.

Analysis of the complete *LMO4* cDNA sequence revealed an open reading frame from nt 781 to 1278, with an ATG codon in the preferred configuration (7) with an A in position -3, and a G in position +4 located at the start of this open reading frame. The amino acid sequence predicted by this open reading frame contains two tandem LIM domain motifs, which conform to the consensus sequence of all known LIM domains (8) and occupy almost the entire length of the 165 amino acid sequence translation product. LIM domains are found in a variety of proteins and describe a cysteine-rich, zinc-binding motif, which interacts with other proteins. Computer homology searches of the nucleic acid data bases detected a 62% identity between the region encoding the two tandem LIM domains, with the analogous region of *LMO1*, a putative oncogene associated with a chromosomal breakpoint in a subset of T-cell leukemias (9,10). The size of the predicted translation product of our isolate (165 bp), is similar to the LMO proteins: LMO1 (160 aa) and LMO2 (158 aa) (9,10). In view of these similarities, we tentatively named the gene of our cDNA isolate, *LMO4*. At the amino acid level, the identity within the LIM domains of LMO1 and LMO4 is 55%, and spacing of the amino acids making up the LIM domains is identical. The LIM domain sequences are so highly conserved that

the identity of LMO4 protein to the LMO1 homologue of Drosophila is just as extensive (54%). A number of the expressed sequence tags found in homology searches were from mouse and rat cDNA libraries and showed identity in a range of 90 to 98%, indicating that *LMO4* is highly conserved in evolution. Although the long untranslated 5' end of *LMO4* cDNA shows a slight homology to some other genes with GC rich 5' end regions, the sequence is unique, as is the long 3' end. *LMO4* does not seem to have any other closely related genes, since in our efforts to obtain full length clones, after extensive hybridization screening of different cDNA libraries, we failed to isolate any related cDNAs.

Northern blot analysis to assess tissue distribution of this gene product revealed it to be present in most tissues analyzed, with highest expression in brain, skeletal muscle, testis and ovary; with little or no expression in liver, kidney and pancreas. Two bands of approximately 2.1 and 1.9 kb could be discerned in most tissue samples however, it is the larger 2.1 kb band which is most prominent in the normal tissue samples, while in breast tumor mRNA the smaller 1.9 kb band is most prominent. This pattern of expression was observed in all breast tumor mRNA samples analyzed. The same two bands hybridized with either a 600 bp 5' end probe, or a probe containing open reading frame and 3' end sequences. Since breast tumors are a complex mixture of different cell types (stromal fibroblasts, infiltrating lymphocytes and transformed breast epithelial cells), the exact source of the *LMO4* transcripts in breast tumors remains to be determined.

A variant *LMO4* cDNA clone, with a 112 base pair deletion in the 5' region (nt 81 - 192), was isolated from different cDNA libraries. This clone probably represents the smaller band observed in northern blots. The deletion alters some possible short open reading frames in the 5' untranslated region, and its role in the expression of LMO4 protein is yet to be determined. Short open reading frames in the 5' untranslated region have been detected in other tightly controlled genes and have been shown to suppress translation.

The extremely long 5' end of *LMO4* cDNA (780 bp) is the GC rich region. A long GC rich, structured 5'-leader sequence is characteristic of transcripts encoding oncoproteins, growth factors, transcription factors, and other regulatory proteins - that seem to be designed to be translated poorly (11). Inhibition at the translational level seems to be a component of gene regulation for genes which need to be tightly regulated. Another feature of the sequence of *LMO4* cDNA is the presence of multiple ATTT motifs in the 3' end, which have also been observed in the 3' untranslated region of numerous lymphokine, cytokine, and proto-oncogene mRNAs. It has been proposed that such ATTT motifs are involved in the selective degradation of transiently expressed messengers (12).

The amino terminal amino acid sequences, immediately preceding the LIM domains of both LMO1 and LMO2 have been shown to be

transactivation domains (13). It has been shown that the four proline residues within the 19 amino acid long activation domain of LMO2 played an important role in conferring full transactivation activity to this domain (13). The amino terminal sequence of the predicted LMO4 protein (MVNPGSSSQPPPVTAGSLSLSW), although not homologous to the analogous sequences of LMO1 or LMO2, is also proline rich and may also be an activation domain.

As a result of our major effort during the past year, we have obtained further evidence for the role and importance of *LMO4* in cellular growth regulation by using the yeast two hybrid screen with an *LMO4* LIM domain construct in a binding domain bait plasmid. We have identified five gene products which were isolated numerous times as activation domain (AD) co-transformants with the *LMO4* bait plasmid. We are now in the process of verifying the authenticity of these two hybrid interactions by biochemical binding assays, immunoassays, as well as individual yeast co-transformations with positive AD clones containing frameshift mutations. All five putative *LMO4*-binding gene products have been sequenced and identified:

1. **Suppressin** - An uncharacterized gene product, identified as a 63 kDa inhibitor of cell proliferation in the Genbank database (# U59659).

2. **Kinesin-2** - (14) A member of a superfamily of motor proteins which are implicated in mechanisms of mitosis or meiosis, and is markedly upregulated in tumor cells after retinoid treatment.

3. **SUPT6H** - (15) An extremely conserved mammalian nuclear protein that regulates transcription through establishment or maintenance of chromatin structure, and appears to play a significant role in the human estrogen receptor signal transduction pathway.

4. **NCS-1** (16) A calcium sensor protein involved in the phosphorylation of components of the signal transduction machinery.

5. **eIF3** An uncharacterized human translation initiation factor.

Although we have not yet verified by biochemical and immunological means the positive interactions of these 5 gene products with *LMO4*, all positive clones were found to have open reading frames in-frame with the AD coding region, supporting the interpretation that the two hybrid interactions represent real affinities. In addition, positive clones from each gene product were not all identical, however they all had portions of their ORFs in frame with the AD coding region. There appears to be a common thread about the function of each of these proteins, which further supports the likelihood that *LMO4* is a vital mediator of differentiation and development, and potentially relevant to malignancy. neither of these five gene products has been fully characterized, nor have they been implicated in any known transcription or other regulatory complexes.

CONCLUSIONS

We have identified and characterized two new gene products (*Ngp-1* and *LMO4*), both of which appear to play vital roles in cellular growth and differentiation. *LMO4* appears to be especially relevant to cellular growth control because of its interaction with other gene products involved in gene regulation and signal transduction. Our observation that the expression of *LMO4* transcripts in breast tumors differs from that in other tissues, and that *LMO4* interacts with a protein involved in estrogen receptor signal transduction, hint at a possible role of *LMO4* in breast cancer, and merits further investigation. Structural features of the *LMO4* cDNA sequence (a long GC-rich structured 5′ end, the presence of mRNA destabilizing motifs in the 3′ end and a predicted amino acid sequence which contains two LIM domain motifs with a partial homology to a known oncogene) all predict that this gene plays a vital role in the life of the organism.

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